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In vivo involvement of mutated initiation factor IF1 in gene expression control at the translational level

Victor V. Croitoru, Margarete Bucheli-Witschel[1], Leif A. Isaksson*

Department of Genetics, Microbiology and Toxicology, Stockholm University, S-10691 Stockholm, Sweden

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Abstract The influence in vivo of mutated forms of translation initiation factor (IF1) on the expression of the *lacZ* or *3A′* reporter genes, with different initiation and/or +2 codons, has been investigated. Reporter gene expression in these *infA*(IF1) mutants is similar to the wild-type strain. The results do not support the longstanding hypothesis that IF1 could perform discriminatory functions while blocking the aminoacyl-tRNA acceptor site (A-site) of the ribosome. One cold-sensitive IF1 mutant shows a general overexpression, in particular at low temperatures, of both reporter genes at the protein but not mRNA level.

Keywords: +2 Codon; IF1; Gene expression; Codon context; Aminoacyl-tRNA acceptor site; *Escherichia coli*

1. Introduction

In prokaryotes, the first steps during translation initiation take place on the 30S ribosomal subunit and require three factors: IF1, IF2-GTP, and IF3, as well as the fMet-tRNA (for reviews, see [1–4]). IF1 binds to the 30S subunit in a 1:1 ratio and this binding is strengthened in the presence of IF2 and IF3 [5,6]. On the other hand, the activities of IF2 and IF3 in the formation of the pre-initiation complex are stimulated when IF1 is present [7,8]. Footprinting and cross-linking studies have indicated that IF1 is associated with IF2, but not with IF3 [9]. The initiation process is finally completed by the association of the 50S ribosomal subunit.

IF1 is encoded by the *infA* gene [10] and is a small basic protein consisting of 71 amino acid residues [11]. It is a member of the oligonucleotide/oligosaccharide binding (OB) fold family of proteins [12], which among others includes RNA-binding proteins such as ribosomal protein S1 [13], the cold shock protein CspA [12,14] and eukaryotic initiation factor (eIF1A) [13,15].

In foot-printing experiments, it has been shown that IF1 binding to the 30S subunit protects bases G530, A1492 and A1493 of the 16S rRNA. These bases are precisely the ones that are protected by the binding of tRNA to the ribosomal aminoacyl-tRNA acceptor site (A-site). It was therefore suggested that IF1 mimics the A-site-bound tRNA and could prevent premature binding of aminoacyl-tRNA by blocking the A-site on the 30S ribosomal subunit [16]. The crystal structure of IF1 bound to the 30S subunit has shown that IF1 indeed occludes the ribosomal A-site and flips out the functionally important bases A1492 and A1493 from helix 44 of 16S RNA, hiding these bases inside IF1 [17]. These latter X-ray data suggest that IF1 binds to the A-site in a different manner than does the A-site-bound tRNA [16,18,19].

The binding of IF1 to the ribosomal A-site raises the possibility that IF1 participates in a fidelity function during initiation, in order to avoid mismatch or frameshift errors at the ribosomal P- and A-sites. Moreover, IF1 has been proposed to prevent premature binding of aminoacyl-tRNA to the A-site during initiation [16]. It is possible that in the presence of IF2, the initiator tRNA is not directed exclusively into the P-site unless the A-site is blocked by IF1 [18].

Even though binding of IF1 to the ribosomal A-site could suggest that IF1 takes part in an initiation fidelity function [20,21], this hypothesis has not been tested so far (latest review [1]). In this study, we have investigated the relationship between IF1 and the nature of the +2 codon immediately following the initiation codon, thus representing the ribosomal A-site. Using some *infA*(IF1) mutant strains [22], we have also addressed the question of any involvement of IF1 in the discrimination against initiation codons other than AUG. Our results do not indicate any involvement of IF1 in recognition of the +2 codon or any changed efficiency of decoding at the near-cognate initiation codons UUG and GUG. Some functional mutants with altered IF1 are generally changed in expression of the two used reporter genes, irrespective of the nature of their +2 codons.

2. Materials and methods

2.1. Chemicals

All chemicals were of the highest grade commercially available. Restriction enzymes and T4 DNA ligase were either from Promega, Fermentas Life Sciences or New England BioLabs. DNA extraction kits were from Qiagen. Plasmids were prepared using a GFX Micro Plasmid Prep Kit from Amersham Pharmacia Biotech. Termination mixtures for automatic sequencing were purchased from Perkin–Elmer.

*Corresponding author. Fax: +46 8 6129552.
E-mail address: leif.isaksson@gmt.su.se (L.A. Isaksson).
URL: http://www.gmt.su.se.

[1] Present address: Department of Environmental Microbiology, EAWAG, CH-8600 Duebendorf, Switzerland.

Abbreviations: IF, translation initiation factor; A-site, aminoacyl-tRNA acceptor site

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2.2. Bacterial strains and plasmids

The *Escherichia coli* MG1655 wild-type and the CVR *infA*(IF1) mutant strains [22] were the hosts for the plasmids [23] used in the *lacZ* and *3A′* assay systems. The plasmids are derivatives of pCMS40 and pHN113, respectively. The 5′ sequence of the 3A′ mRNA is: 5′AAUUGUGAGCGGAUAACAAUUUCACCAGGUAAUAAA-UUAAAUAAAAUUUAAAUAUGNNN...-3′ for gene variants lacking a Shine–Dalgarno sequence where NNN corresponds to the +2 codon. For gene variants carrying the Shine–Dalgarno sequence, the region is the same except that the underlined sequence is: AAG-GAGG. Strains containing a chromosomal deletion of the *lacZ* gene were used with appropriate *lacZ* carrying plasmids for the β-galactosidase assays.

2.3. β-Galactosidase assays and growth conditions

Wild-type MG1655 and CVR *infA*(IF1) mutants were grown overnight at 37 °C in M9 minimal medium supplemented with all amino acids at recommended concentrations and 100 μg/ml ampicillin ([24] and references therein). Such cultures were used to inoculate into the same medium at 37 °C. Alternatively, cells in LB medium were grown at 30 °C.

Exponentially growing cells (OD_{540} of 0.4–0.5) were harvested without IPTG induction, since the *trc* promoter is leaky, giving significant expression even in the absence of induction. β-Galactosidase activity of the lysed uninduced cells was then determined as described earlier ([24] and references therein). All measurements were carried out using an iEMS multiscan microplate photometer (Labsystems).

2.4. Cell growth and protein A′ purification

The protein A′ assay system is based on the IgG binding protein A from *Staphylococcus aureus* (*S. aureus*) as a reporter gene, where A′ is the IgG binding domain [25]. The different +2 codons were cloned into a reporter gene that encodes three A′ (3A′) domains. Codon context dependent expression was measured for the 21 kD 3A′ protein and related to a constitutively expressed internal control 2A′ gene (producing a 14 kD protein with two A′ domains) in the same plasmid. Both genes are under separate control of a *trc* promoter. Strains with appropriate 3A′-carrying plasmids were cultured overnight at 30 °C in LB medium supplemented with ampicillin (100 μg/ml). A 100-fold dilution was used as inoculum for growth in the same medium and growth was followed by spectrophotometer measurements. For induction, IPTG was added at $OD_{590} = 0.2$ in the mid-log phase of growth. When the cells reached an $OD_{590} = 0.5$, they were cooled on ice and harvested by centrifugation, followed by re-suspension in 1 ml of 10× TST buffer (0.5 M Tris, pH 7.4, 2.5 M sodium chloride, and 0.5% Tween 20) [25]. Cells were lysed by incubation at 95 °C for 10 min and cell debris was eliminated by centrifugation. The 3′A test protein and the 2′A control protein were co-purified from the supernatant fraction using IgG–Sepharose (Pharmacia) mini-columns and a vacuum mini-fold system (Promega). The 3A′ and 2A′ proteins were eluted with 0.1 ml of 0.5 M acetic acid at pH 3.2. The eluant was dried in a vacuum Speed-Vac (Techtum). Protein samples were dissolved in sample loading buffer, after denaturation at 95 °C for 2 min. Analyses of the A′ proteins were done by using SDS–PAGE, and protein bands were scanned and quantified as described previously [25]. The molar ratio of the two bands 3A′/2A′ was used to evaluate the effect of the +2 codon on gene expression.

2.5. Preparation and Northern blot analysis of mRNA levels

Total cellular RNA from *E. coli* wild-type and CVR mutant strains with plasmids carrying specific +2 codons in the *3A′* test gene system was prepared by using the RNEasy kit (Qiagen). 0.1 mM IPTG was added at $OD_{590} = 0.2$ and the 3 ml cultures were harvested in mid-log phase of growth at $OD_{590} = 0.5$. RNA samples were separated on 1% agarose gels and transferred to a Hybond-N nylon membrane (Amersham Life Science). The transferred RNA was hybridized 16–20 h with the ^{32}P-labelled deoxyoligonucleotide probe ABP01 (5′-CGTTGTTCTTCGTTTAAGTTAGG-3′), which is complementary to each of the A′-encoding RNA sequences. The radioactive signals on the films were analyzed by scanning (FujiFilm FLA-3000).

3. Results

3.1. Experimental strategy

It has been shown that the nature of the +2 codon, that follows the initiation codon, markedly influences gene expression [23,26]. However, no explanation for this phenomenon has been formulated yet. Since IF1 is situated near the +2 codon during translation initiation, it is possible that IF1 might interact with this codon, thus contributing to the observed effect. In order to investigate this possibility, we have used some *lacZ* and *3A′* reporter gene constructs containing various codons at the +2 position [23]. We introduced them into chromosomal *infA*(IF1) mutants, containing specific arginine to leucine exchanges at different positions [22], and measured expression of a *lacZ* or *3A′* reporter gene with various +2 codon alterations, or near-cognate initiation codons, in these mutant strains. The purpose of the experiments was to analyze if any general or codon specific difference in the expression pattern of the reporter gene could be obtained by using diverse *infA* mutants that are altered in their phenotypes, compared to the wild-type *E. coli* strain MG1655.

3.2. The effect of IF1 alterations on the expression of lacZ and 3A′ reporter genes with various +2 codons

Fourteen reporter constructs lacking any Shine–Dalgarno sequence and varying only in the +2 codon [23] were tested in six chromosomal *infA*(IF1) mutants with arginine to leucine alterations [22]. In our model system, in agreement with an earlier report [23], the gene expression levels in the wild-type strain varied by a factor of about 25-fold, depending on the +2 codon. Also, the construct carrying the AAA codon at +2 gave the highest expression level, while the GGG codon at this position gave the lowest gene expression (Table 1).

No significant effects of the mutationally altered IF1 on the expression pattern associated with the +2 codons were observed when compared to the wild-type strain (Table 1). Nevertheless, a slight upregulation of the *lacZ* reporter gene expression was indicated in the cases of the CVR65L and CVR69L mutant strains grown at 37 °C. In contrast, a slight downregulation of gene expression was observed in the case of the CVR71L mutant (Table 1). Previously, it has been reported that the CVR69L mutant grows slower than wild-type, in particular at temperatures below 30 °C, and is therefore partly cold-sensitive [22]. This property at low temperature is especially pronounced in a medium supporting fast growth, such as LB medium. In line with these results, most reporter gene variants in the cold sensitive mutant CVR69L were increased in expression, compared to gene expression in the wild-type strain at 30 °C (Table 2), being almost twofold in LB medium. However, at 37 °C the difference in gene expression was barely significant, even in LB medium, if wild-type and CVR69L cells were compared (data not shown). The temperature dependent behavior of the CVR69L mutant was compared to that of the CVR22L mutant, which displays growth rates similar to the wild-type [22]. In this case, the gene expression level of CVR22L was equal to wild-type in both growth media (LB and M9), independently of the temperature (data not shown). This fact emphasizes the generally elevated gene expression at low temperature observed in the cold-sensitive CVR69L strain, in contrast to what is seen for the CVR22L mutant and

Table 1
Effect of different A-site codons (+2 codons) on the expression of *lacZ* gene variants in *infA*(IF1) mutants

Plasmid	A-site codon	Relative gene expression						
		MG1655	CVR22L	CVR40L	CVR45L	CVR65L	CVR69L	CVR71L
pCMS 40	AAA	1.0 (122)	1.18 ± 0.01	0.96 ± 0.02	1.18 ± 0.03	1.02 ± 0.06	0.96 ± 0.07	0.76 ± 0.04
pCMS 77	AGA	1.0 (99)	1.22 ± 0.04	1.07 ± 0.02	0.88 ± 0.02	1.19 ± 0.08	1.09 ± 0.09	0.97 ± 0.09
pCMS 76	CGA	1.0 (31)	1.15 ± 0.01	1.04 ± 0.04	0.87 ± 0.03	1.43 ± 0.16	1.19 ± 0.11	0.79 ± 0.06
pCMS 132	CGC	1.0 (33)	1.26 ± 0.01	1.02 ± 0.04	1.20 ± 0.06	1.97 ± 0.20	1.80 ± 0.13	0.88 ± 0.07
pCMS 130	CGU	1.0 (62)	1.09 ± 0.02	1.13 ± 0.08	0.88 ± 0.03	1.20 ± 0.06	1.24 ± 0.11	0.91 ± 0.10
pCMS 132	CGG	1.0 (9)	1.38 ± 0.02	1.12 ± 0.01	0.91 ± 0.04	1.84 ± 0.15	1.43 ± 0.10	0.74 ± 0.04
pCMS 133	AGG	1.0 (17)	1.08 ± 0.01	1.03 ± 0.02	0.82 ± 0.03	1.72 ± 0.11	1.12 ± 0.12	0.55 ± 0.09
pCMS 104	GAC	1.0 (47)	0.96 ± 0.04	1.41 ± 0.09	1.08 ± 0.03	0.97 ± 0.10	0.90 ± 0.07	0.87 ± 0.08
pCMS 100	UUC	1.0 (43)	1.34 ± 0.03	1.33 ± 0.02	0.76 ± 0.04	1.70 ± 0.15	1.59 ± 0.12	0.95 ± 0.11
pCMS 75	AAG	1.0 (70)	1.15 ± 0.01	1.19 ± 0.01	1.18 ± 0.09	1.51 ± 0.06	1.23 ± 0.08	0.91 ± 0.05
pCMS 70	GAA	1.0 (8)	0.86 ± 0.01	0.98 ± 0.02	1.07 ± 0.01	1.92 ± 0.08	1.24 ± 0.09	0.77 ± 0.06
pCMS 113	UGG	1.0 (17)	0.89 ± 0.03	0.96 ± 0.02	0.97 ± 0.02	1.63 ± 0.11	1.51 ± 0.12	0.73 ± 0.08
pCMS 99	GAG	1.0 (7)	1.14 ± 0.01	1.04 ± 0.01	0.95 ± 0.01	1.79 ± 0.16	0.99 ± 0.06	0.89 ± 0.09
pCMS 129	GGG	1.0 (5)	1.08 ± 0.01	1.18 ± 0.01	0.85 ± 0.01	1.20 ± 0.06	1.22 ± 0.08	0.77 ± 0.09

Cells were grown at 37 °C in M9 minimal medium supplemented with amino-acids. Absolute values for the wild-type strain are shown in parentheses in Miller units. Expression in the CVR mutants in the case of each indicated codon is given relative to the corresponding value in MG1655. Relative values and standard errors are shown. The *lacZ* SD$^-$ gene variants were used. Plasmid details are as described in Section 2.

Table 2
Medium effects at 30 °C on A-site codon (+2 codon) influences on the expression of *lacZ* gene variants

Plasmid	A-site codon	Relative gene expression			
		M9		LB	
		MG1655	CVR69L	MG1655	CVR69L
pCMS 40	AAA	1.0 (85)	1.03 ± 0.11	1.0 (42)	1.76 ± 0.11
pCMS 77	AGA	1.0 (58)	1.34 ± 0.01	1.0 (32)	1.82 ± 0.13
pCMS 76	CGA	1.0 (25)	1.36 ± 0.07	1.0 (12)	1.84 ± 0.20
pCMS 132	CGC	1.0 (22)	1.63 ± 0.06	1.0 (9)	1.98 ± 0.21
pCMS 130	CGU	1.0 (29)	1.71 ± 0.05	1.0 (13)	2.05 ± 0.10
pCMS 132	CGG	1.0 (10)	1.12 ± 0.02	1.0 (5)	1.45 ± 0.16
pCMS 133	AGG	1.0 (7.6)	1.16 ± 0.02	1.0 (6)	1.36 ± 0.10
pCMS 104	GAC	1.0 (29)	1.14 ± 0.04	1.0 (15)	1.49 ± 0.11
pCMS 100	UUC	nd	nd	1.0 (8)	1.93 ± 0.19
pCMS 75	AAG	1.0 (37)	1.32 ± 0.06	1.0 (17)	1.92 ± 0.09
pCMS 70	GAA	nd	nd	1.0 (3)	1.78 ± 0.04
pCMS 113	UGG	nd	nd	1.0 (7)	1.79 ± 0.14
pCMS 99	GAG	nd	nd	1.0 (3)	1.83 ± 0.14
pCMS 129	GGG	nd	nd	1.0 (3)	1.46 ± 0.21

Absolute values for the wild-type strain are shown in parentheses in Miller units. MG1655 and the cold sensitive CVR69L mutant were grown in LB or M9 medium supplemented with amino-acids. Plasmid details, *lacZ* gene variants and relative expression presentations are as explained in Table 1. nd, not determined.

wild-type strains (Table 2). Higher gene expression was also observed at low temperature in the case of another IF1 mutant with an Arg to Asp alteration (data not shown).

Using the *3A′* reporter system, we observed a similar overexpression pattern also for this test gene when monitored in the CVR69L mutant in LB medium at 30 °C (Table 3), even though this effect was slightly less pronounced when compared to the *lacZ* test system (Table 2). In contrast to the CVR69L mutant, the CVR71L strain displayed a slightly lower expression level of the *3A′* reporter gene in LB medium at 30 °C (Table 3). This is in line with the results obtained for *lacZ* expression in the CVR71L mutant in minimal medium at 37 °C (Table 1).

The possibility that different expression levels in the CVR69L and CVR71L mutants were the result of different mRNA pools was analyzed. As shown in Fig. 1, the mRNA levels were similar for some of the analyzed *3A′/2A′* reporter gene variants used in this study. On the contrary, 3A′ protein levels, relative to the internal control 2A′, were increased in the cases of both analyzed +2 codons in CVR69L (Table 3). This suggests that overexpression of the *3A′* test gene in CVR69L takes place at the translational level, presumably during the

Table 3
A-site codon (+2 codon) effects on the expression of *3A′* reporter gene variants

Plasmid	A-site codon	Relative gene expression (3A′/2A′)		
		MG1655	CVR69L	CVR71L
pHN113	AAA	1.0 (0.17)	1.54 ± 0.02	0.89 ± 0.01
pHN118	AGA	1.0 (0.24)	1.36 ± 0.01	0.92 ± 0.01
pHN117	CGA	1.0 (0.08)	1.18 ± 0.07	0.97 ± 0.06
pHN333	CGC	1.0 (0.05)	1.17 ± 0.05	0.66 ± 0.03
pHN335	AGG	1.0 (0.02)	1.96 ± 0.03	1.05 ± 0.03

The wild-type strain MG1655, the *infA*(IF1) mutants CVR69L and CVR71L were grown at 30 °C in LB medium. Values in brackets represent the 3A′/2A′ molar ratios [25]. This protein expression value for the CVR mutants is given relative to the same analyzed +2 codon in MG1655. SD$^-$ variants of the *3A′* reporter genes with indicated A-site (+2 codons) were used.

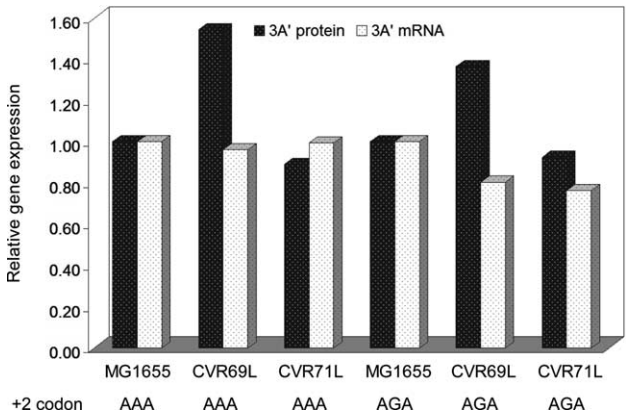

Fig. 1. Expression of the 3A′ test gene at the protein and mRNA levels. The 3A′ gene variants are SD^- with indicated +2 codon. Expression values for the indicated *infA*(IF1) mutants are given as compared to the same codon in the wild-type strain, defined as 1.0, as explained in Table 3. The standard error of all experiments is ±0.1 or less.

initiation phase. The results obtained for the CVR71L mutant were less indicative.

3.3. The effect of IF1 alterations on the expression of lacZ genes with different initiation codons

To test for any influence of the altered IF1 on initiation at different initiation codons, *lacZ* constructs with AUG, GUG or UUG initiation codons were tested in the context of some different A-site (+2) codons (Table 4). In a wild-type strain, these initiation and +2 codons have been analyzed earlier [27]. Also, the extremely weak initiation codon AUU was analyzed, but the expression values were too low to allow for a reliable interpretation (data not shown). As shown previously, AUG is the most efficient start codon, UUG is intermediate and GUG is weak in the test system used here [27]. If IF1 is involved in discrimination of the initiation codon, a changed expression from various initiation codons could possibly be expected in the case of some IF1 mutants. However, as can be seen in Table 4, the CVR69L mutant gave twofold increased expression levels for all tested initiation codons, when compared to the wild-type strain, in line with results discussed above (Table 2). This increase was independent of the initiation codon context. No significant effect on gene expression was observed for any of the five other IF1 mutants, also having Arg to Leu alterations [22], compared to wild-type strain (data not shown). Also for these mutants, all analyzed near-cognate initiation codons were less efficient than the canonical AUG initiation codon, thus resembling the situation for the wild-type strain [28,29].

Table 4
Gene expression of *lacZ* constructs, starting with different initiation codon contexts, in the cold sensitive CVR69L mutant

Plasmid	P-site/A-site codons	Relative gene expression	
		MG1655	CVR69L
pCMS 30	AUG AAA	1.0 (182)	1.9 ± 0.06
pCMS 17	UUG AAA	1.0 (129)	2.0 ± 0.04
pCMS 207	GUG AAA	1.0 (51)	1.7 ± 0.01
pCMS 352	AUG AGU	1.0 (156)	1.8 ± 0.05
pCMS 354	AUG AAU	1.0 (266)	1.3 ± 0.04
pCMS 353	AUG ACC	1.0 (176)	1.9 ± 0.04
pCMS 343	UUG AGU	1.0 (123)	2.0 ± 0.02
pCMS 345	UUG AAU	1.0 (125)	1.5 ± 0.02
pCMS 344	UUG ACC	1.0 (80)	1.5 ± 0.01
pCMS 500	GUG AGU	1.0 (35)	2.0 ± 0.01
pCMS 502	GUG AAU	1.0 (48)	1.9 ± 0.01
pCMS 501	GUG ACC	1.0 (31)	1.7 ± 0.01

Cells were grown at 30 °C in LB medium. Absolute values for the wild-type MG1655 are shown in parentheses in Miller units. SD^+ variants containing a canonical Shine–Dalgarno sequence (AAGGAGG) of *lacZ* were analyzed. Relative values and standard errors are shown. Plasmid details and expression calculations are as described in Table 1.

4. Discussion

It has been hypothesized that IF1 could influence the initiation fidelity while binding to the ribosomal A-site during initiation [16,30]. Based on our results using several *infA*(IF1) mutants and expression of two different model genes with various +2 codons in the mRNA, we suggest that IF1 has no codon discriminatory functions with respect to the +2 codon, or to the nature of the initiation codon (AUG, UUG or GUG), while occluding the A-site. This suggestion is in line with the fact that interaction of IF1 with the 30S subunit is mainly electrostatic [5,22] and no specific interaction between IF1 and bases in mRNA has been detected so far. Cross-linking studies have shown that IF1 binds to mRNA in vivo, although it is not clear if this interaction takes place during initiation of translation or reflects the activities of non-ribosomal associated IF1 [31,32]. In an early work, IF1 was shown to alter the structure of various oligonucleotides by disrupting nucleic acid interactions in vitro [33], but no specific sequence requirements were found. Thus, a codon-unspecific response of IF1 mutant variants to the codon located in the A-site reported in this study is in agreement with previous data.

It is known that IF1 is an RNA binding protein being a member of the oligonucleotide/oligosaccharide binding fold (OB-fold) family of proteins [12], which among others includes RNA-binding proteins such as the cold shock protein CspA [14] and eIF1A [13,15]. IF1 displays a striking structural resemblance to the major cold shock protein CspA [34,35]. It has also been shown that heterologous expression of IF1 from *E. coli* in a *Bacillus subtilis cspB/cspC* double deletion strain complemented the loss of the cold shock induced protein CspB in *B. subtilis* (the homolog of CspA in *E. coli*) [36]. This finding led to a conclusion that IF1 and cold shock proteins have overlapping functions [36] and they could have evolved from a common ancestor [37]. Recently, it was shown that IF1 and CspA preferentially stimulate translation at low temperatures without mRNA selectivity [38] and that cold shock induces a stoichiometric imbalance between initiation factors and ribosomes [39]. Our finding that the R69L alteration in IF1 causes a twofold increase in gene expression at low temperature, as well as cold-sensitivity, suggests that IF1 is an active player in the initiation complex, not only passively stimulating the activities of IF2 and IF3. This implication is supported by our finding that another cold-sensitive *infA* mutant, having an Arg to Asp alteration, shows even higher overexpression of both the *lacZ* and *3A′* reporter genes (data not shown). Our results correlate with a recent finding that IF1 stimulates translation of several different mRNAs by 30–40% in the cold [38]. Since the mRNA levels for the *3A′* test gene are unaffected

by the *infA* mutations, this strongly suggests that the increased reporter gene expression seen in the case of the cold-sensitive CVR69L mutant (Table 3, Fig. 1) arises at the translational level.

The change from a positive to neutral charge due to the replacement of the Arg69 with leucine in the mutant CVR69L gives increased expression of both the *lacZ* and *3A′* based reporter systems. Even if the effect is being only twofold, it is consistent. If many *E. coli* genes are similarly increased in expression, this could result in higher stress on the translational apparatus. This could lead to a slow growth at low temperatures, thus explaining the slow growth observed for the CVR69L mutant. Further in vitro analysis is performed to define this cold-sensitive behavior.

The amino-acid residue Arg69 has been shown to be a part of the 30S binding site and deletion of the last three residues in IF1 (Arg69–Ser70–Arg71) results in substantially lowered affinity of IF1 for the 30S subunit [40]. In addition, this version of IF1 with the C-terminal deletion shows a much reduced biological activity in promoting 30S initiation complex formation. This finding suggests that the C-terminus of IF1 is a functionally important part of the factor and its positive charge distribution is crucial for the function of IF1 [22]. In the same study, it was also reported that the double alteration R69L + R71L in IF1 is even more cold-sensitive and is unable to grow below 20 °C. Furthermore, in this case the corresponding *infA* allele must be present in a multi-copy plasmid for cell survival, since this altered gene does not give a viable strain if present in the chromosome only [22]. Also, this finding underlines the functional importance of the C-terminal end of IF1.

The IF1 mutants studied here are functionally altered as revealed by their growth phenotypes [22]. Nevertheless, this defect is neither reflected in any altered sensitivity to the nature of the initiation codon nor to the nature of the +2 codon that follows it. Our results suggest that IF1 is not involved in any recognition of the initiation codon context. However, data obtained for one of the mutants (CVR69L) indicate that IF1 in some way influences gene expression at the level of translation. This phenomenon is under further investigation.

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